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(54) **Moraxella bovis bacterial.**

(57) Protease produced by *Moraxella bovis* can be used as an immunoprophylactic agent for protection against infection by *M. bovis*.

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Moraxella bovis Bacterin

FIELD OF THE INVENTION

This invention relates to veterinary vaccines and, in particular, to a Moraxella bovis bacterin.

BACKGROUND OF THE INVENTION

Moraxella sp. belong to the Family Neisseriaceae. They are strictly aerobic, gram negative, plump rods in pairs or short chains and are oxidase (+) and catalase (+). They are pathogenic in mammals, causing conjunctivitis, sometimes referred to as pink eye.

Bijsterveld, Amer. J. Ophthamology 72 (1):181-184 (1971), reports that two species isolated from human clinical infections, M. liquefaciens and a new carbohydrate-splitting species, produce different types and amounts of proteases.

Moraxella bovis is the etiologic agent of infectious bovine keratoconjunctivitis (IBK), sometimes referred to as bovine pinkeye. Baptista, Br. Vet. J. 135:225-242 (1979), reviewed the incidence, symptoms, etiology, treatment and control of IBK.

Frank and Gerber, J. Clin. Microbiol. 13(2):269-271(1981), report that M. bovis produces tissue damaging enzymes which may initiate or potentiate IBK.

Pugh et al., Canad. J. Comp. Med. 37:70-78 (1973), report a role for M. bovis toxins in reactogenicity of live M. bovis vaccines.

Henson and Grumbles, Cornell Vet. 51:267-284 (1961), report production by M. bovis of a hemolytic toxin and a dermonecrotic toxin.

SUMMARY OF THE INVENTION

The invention resides in the discovery that proteolytic enzymes produced by Moraxella bovis can be used as an immunoprophylactic agent for prevention of IBK. More particularly, one aspect of the invention is a vaccine capable of inducing immunity to Moraxella bovis without serious side effects comprising a vaccinal amount of M. bovis protease.

Another aspect of the invention is a vaccine capable of inducing immunity to M. bovis without serious side effects comprising a vaccinal amount of a M. bovis bacterin which contains a component having proteolytic activity.

DETAILED DESCRIPTION OF THE INVENTION

Moraxella bovis strains useful in preparing the vaccine of the invention can be isolated from clinical cases of IBK or can be obtained from available sources. Available sources include, for example, the American Type Culture Collection in Rockville, Maryland, U.S.A., where M. bovis strains are deposited under accession numbers 10900, 17947 and 17948. The bacteria will grow on most common bacterial culture media. However, to prepare the vaccine of the invention, the bacteria is grown in a medium in which the bacteria will produce M. bovis protease as the presence of M. bovis protease in the vaccine is critical.

Table 1, below, illustrates the criticality of the presence of M. bovis protease in the vaccine by showing the relationship of protease activity of various bacterin

suspensions to protection f[illegible]le and mice against experimental challenge wi[illegible]ent M. bovis.

Protease activit[illegible] of [illegible] bacterin was measured in Trypticase Soy Agar plate[illegible]ining 0.5% autoclaved skim milk. Ten microliters of bac[illegible]rin were added to 3 mm wells. The zones of milk pr[illegible]olysis were measured after 24 hours by first tracing a [illegible] p[illegible]ection of the zone and then measuring the zone of proteol[illegible]s with a planimeter. The area measured by the plan[illegible]e[illegible] was designated as Units of Protease Activity. Relat[illegible]e [illegible]tease activity (RPA) is defined as:

$$\frac{\text{Proteolytic unit[illegible] o[illegible]st bacterin}}{\text{Proteolytic unit[illegible] o[illegible]ference bacterin}} = \text{RPA}$$

The relative pot[illegible]c[illegible] of a bacterin was determined as follows: Bacterins we[illegible]uted 5-fold in 0.15 M NaCl. Mice (16-20 grams) were v[illegible]c[illegible]ed twice, intraperitoneally (IP) at 21-day intervals wit[illegible] 5 ml of an appropriate dilution. At least three [illegible] dilutions of bacterin were made with the range selected [illegible] the lowest dilution would protect 50% of the vaccinated mice. Mice were challenged IP 7 days following the second vaccination with 0.5 ml dose containing 5-50 LD_{50} of virulent M. bovis. All survivors in each test dilution were recorded 3 days following challenge. The 50% protective endpoint dilution (PD_{50}) of the bacterin was determined by the method of Reed and Muench, Amer. J. Hygiene 27:93-497 (1938). The relative potency (RP) of a bacterin is the ratio of the PD_{50} of that bacterin to the PD_{50} of the reference bacterin. The reference bacterin used herein was M. bovis strain Neb-9, grown in culture medium number 4, Table 2, below, for about 9.5 hours at about 33°C, substantially as described below.

Table 1

Vaccination Status	Relative Protease Activity of Bacterin	Relative Potency of Bacterin in Mice	No. of Calves Protected from IBK/No. of Calves Challenged (% protected)
+	0.40 - 0.94	.08 - 1.10	22/29 (70%)
+	0.21	0.04	1/7 (14%)
-	0	0	1/20 (5%)

M. bovis protease production is greatest when it is grown in enriched media. For example, Table 2 illustrates the effect of growth medium composition on protease production of M. bovis. These results, and the results of another experiment shown in Table 3, below, show that protease production is enhanced by addition to the growth medium of a substrate which induces protease production. Examples of such substrates include, but are not limited to, casein or a casein digest; hyaluronic acid, chondroiten sulfate or other tissue constituents contributing to tissue integrity; yeast extract; beef infusion; and tryptone. Other such substrates can be readily identified by testing cells grown in the presence of such substrate for proteolytic activity as described above. Hyaluronic acid and chondroiten sulfate and other factors play a role in binding cells to preserve tissue integrity; the activity of the M. bovis protease appears to be directed at such cell binding.

The results show that protease production is especially enhanced by addition of a source of casein, such as a Milk Stock, (or a casein digest such as N-Z Amine A) to the growth medium. For example, compare media 1 and 5, Table 2 and compare media 7 and 8, Table 3.

Medium	Units of Protease Activity (Proteolytic Units)	Relative Protease Activity
1. RPMI - 1640 .2% Sodium bicarbonate	0	0.00
2. BME Earle's Powder 10% Fetal Bovine Serum Bovine Corneal Cells	890	0.40
3. Eugon Broth (360 g) Yeast extract (60 g) Tween 85 (600 ml) Tween 40 (300 ml) Milk Stock (600 ml) .04% Chondroitin Sulfate (960 ml) .02% Hyaluronic Acid (2,400 ml) Water (7,140 ml)	1634	0.73
4. Eugon Broth (360 g) Yeast Extract (60 g) Tween 85 (500 ml) .04% Chondroitin Sulfate (960 ml) .02% Hyaluronic Acid (2,400 ml) Milk Stock (600 ml) Water (9800 ml)	2222	1.00
5. RPMI - 1640 (10 L) .2% Sodium Bicarbonate N-Z Amine A (200 g)	2536	1.14

RPMI-1640 is a product of Grand Island Biological Company, Grand Island, New York. It contains the following ingredients (mg/L):

$Ca(NO_3)_2$ $4H_2O$ (100)	L-methionine (15)
KCl (400)	L-phenylalanine (15)
$MgSO_4$ (48.84)	L-proline (20)
NaCl (6000)	L-serine (30)
Na_2HPO_4 (800)	L-threonine (20)
glucose (2000)	L-tryptophane (5)
glutathione (red.) (1)	L-tyrosine (28.94, Na salt)
L-arginine (free base) (200)	L-valine (20)
L-asparagine (50)	biotin (.20)
L-aspartic acid (20)	D-Ca pantothenate (.25)
L-cystine (65.15, 2 HCl)	choline Cl (3)
L-glutamic acid (20)	folic acid (1)
L-glutamine (300)	i-inositol (35)
glycine (10)	nicotinamide (1)
L-histidine (free base) (15)	p-aminobenzoic acid (1)
L-hydroxyproline (20)	pyridoxine HCl (1)
L-isoleucine (allo free) (50)	riboflavin (.20)
L-leucine (met-free) (50)	thiamine (1)
L-lysine HCl (40)	vitamin B12 (.005)

BME Earle's Powder is a product of the Grand Island Biological Company, Grand Island, New York. It contains the following ingredients (mg/L).

$CaCl_2$ (200)	L-phenylalamine (16.50)
KCl (400)	L-threonine (24)
$MgSO_4$ (anhyd.) (97.67)	L-tryptophane (4)
NaCl (6800)	L-tyrosine (26)
NaH_2PO_4 H_2O (140)	L-valine (23.50)

glucose (1000)		biotin (1)
phenol red (10)		D-Ca pantothenate (1)
L-arginine HCl (21)		choline chloride (1)
L-cystine 2HCl (15.6		folic acid (1)
L-glutamine (292)		i-inositol (2)
L-histidine (8)		nicotinamide (1)
L-isoleucine (26)		pyridoxal HCl (1)
L-lysine HCl (36.47)		riboflavin (.10)
L-leucine (26)		thiamine HCl (1)
L-methionine (7.5)		

Hugon Broth is a product of PBL Microbiology Systems, Cockeysville, Maryland. It contains the following ingredients (mg/L):

trypticase peptone (5)	sodium sulfite (.2)
phytone peptone (5)	L-cystine (.7)
NaCl (4)	dextrose (5.5)

N-Z Amine A is a casein digest sold by Sheffield Products, Norwich, New York

Culture medium number 5, Table 2, is herein referred to as the "bacterin medium." It is the preferred medium for production of the bacterin and the protease of the invention.

Results of a similar experiment are reported in Table 3.

TABLE 3

Medium	Relative Protease Activity
1. Plate Count Broth (4.75 g) 0.5% Yeast Extract (1.25 g) Water (250 ml)	336
2. Plate Count Broth (4.25 g) Water (250 ml)	420

TABLE (CONT'D)

3.	Mueller-Hinton Broth (3.8 g) Water (250 ml)	423
4.	Mueller-Hinton Broth (3.8 g) 0.5% Yeast Extract (1.25 g) Water (250 ml)	465
5.	MIE Medium (237.5 ml) Bacto B (5 ml)	600
6.	RPMI-1640 (245 ml) Bacto B (5 ml)	649
7.	Eugon Broth (237.5 ml) 0.5% Yeast Extract (1.25 g) 5% Tween 85 (12.5 ml)	788
8.	Eugon Broth (225) 0.5% Yeast Extract (1.25 g) 5% Milk Stock (12 ml) 5% Tween 85 (12.5 ml)	1271

Plate Count Broth is a product of Difco Laboratories, Detroit, Michigan. It contains 5 g of yeast extract, 10 g of tryptone and 2 g of dextrose per liter of water.

Mueller-Hinton Broth is described by Meuller et al., Proc. Soc. Exp. Biol. Med. 48:330 (1941). It contains 300 g of beef infusion, 17.5 g of Acidicase peptone and 1.5 g of starch per liter of water.

MIE medium contains the following ingredients (mg/L).

L-cystine (200)	serine (100)
tyrosine (200)	uracil (100)
leucine (300)	hypoxanthine (20)
arginine (340)	inosine (2000)
glycine (300)	K_2HPO_4 (diab.) (3480)
lysine (5)	KH_2PO_4 (anhy.) (2720)
methionine (100)	yeast extract (10000)

Bacto Supplement B is a product of Difco Laboratories, Detroit, Michigan, is an enrichment for use in

supplementing media. It comprises accessory growth factors of fresh yeast. It also contains glutamine coenzyme (v factor), a nanoxylase and other growth factors.

Protease production is also dependent on duration of growth. Table 4, which follows, shows relative protease production of a strain of M. bovis cultured for different lengths of time in the broth medium.

TABLE 4

Time (hrs)	Colony Forming Units/ml	Relative Protease Activity
0	2.4 x 10^{4}	0
3	1.3 x 10^{7}	650
4	3.0 x 10^{7}	788
5	5.0 x 10^{7}	800
6	2.3 x 10^{8}	1037
8	7.0 x 10^{8}	1280
24	1.4 x 10^{9}	1660

Protease production can also vary depending upon the strain of M. bovis employed. For example, under substantially identical conditions of growth, strain NEB-9 produced 2217 proteolytic units, strain FLA-64 produced 1846 proteolytic units and strain ATCC 10900 produced 900 proteolytic units.

A vaccine against M. bovis can be prepared from the protease, preferably isolated from the culture medium. More preferably, however, the protease is administered in a bacterin comprising killed M. bovis cultured under conditions which promote protease production, such as hereinabove described. Such bacterin preferably contains at least sufficient protease to provoke an immune response, that is, to stimulate production of antibody, to the protease.

Typically, a M. bovis seed stock is inoculated into

a bacterin medium, as described above. The culture is incubated at 30 to 35°C, preferably 33°C, for 8 to 24 hours with aeration. Following satisfactory growth, the culture is transferred to fresh medium using, for example, a 1 to 5% (vol/vol) inoculum. This second seed passage containing dihydrostreptomycin at a final concentration of 0.01% is cultured at 30 to 35°C, preferably 33°C, for 16 to 30 hours with aeration.

Production cultures are prepared by inoculating a medium with actively growing cells, for example, a 1 to 5% (vol/vol) inoculum of the second seed passage. Such culture is aerated to maintain high oxygen content, preferably at least about 80% dissolved oxygen. The pH is maintained at neutral to slightly alkaline, for example, pH 7.3, by addition of base, for example, 5N NaOH. The culture is incubated at 30 to 35°C, preferably 33°C, for at least 2 hours, preferably 4 to 24 hours, until absorbance at 590 nm is at least 2.0 absorbance units, preferably at least 4.0 absorbance units.

After determining cell density and confirming purity, aeration is discontinued, agitation is slowed and temperature is decreased to below 30°C, preferably to about 25°C. The culture is then inactivated by addition of a known inactivating agent, such as, for example, formaldehyde or gluteraldehyde. The preferred inactivating agent is beta-propiolactone (BPL) at a final concentration of 1:1200 (0.083%) because BPL has been found to be rapidly effective. Inactivation is continued until complete, usually about 2 to 10 hours.

The inactivated culture may be stored at 4°C until used. A preservative, for example, 10% merthiolate at a final concentration of 1:10,000, is added. The bacterin is adjuvanted with a known adjuvant, for example, $Al(OH)_3$ or Carbopol (Carbomer, Goodrich) The preferred adjuvant is Quil A at 0.5 mg/ml. Quil A is a saponin. See, Dalsgaard,

Acta Veterin. Scand. Supp 9:1-40 (1978).

The bacterin is standardized to contain not less than 2.0 absorbance units at 590 nm, and, preferably to contain 4.0 absorbance units at 590 nm, by dilution, if necessary, with, for example, saline. Such dosage unit approximately corresponds to a relative potency (RP), as defined above, of 0.4 or greater.

Alternatively cells can be removed from the culture medium, before or after inactivation, and the crude supernatant which contains the protease can be employed as the immunoprotective agent. Preferably, however, in this alternative procedure, the protease is purified by standard protein purification techniques, such as by chromatography, and the purified protease is employed as the immunoprotective agent. The protease is adjuvanted and administered in units of relative potency of 0.4 to greater than 1.0, preferably greater than 1.0.

The vaccine of the invention is administered, preferably, in two 2.0 ml doses subcutaneously in the neck region of calves, three weeks apart. Higher and lower doses, depending, for example, on animal size and relative potency of the vaccine, and other routes and schedules of administration can be used. For example, dose volumes of 1 to 3 ml can be administered intramuscularly or subcutaneously around the eyes.

Primary immunization of calves should be initiated at 4 weeks of age and a booster dose given 3 weeks later. Annual revaccination is recommended.

The following Examples of the invention are illustrative and not limiting.

EXAMPLE 1

Master Seed Stock and Challenge Cultures

M. bovis was isolated from a calf with IBK. The isolate was passed twice on Trypticase Soy Agar containing

0.5% sheep red blood cells (RBC). The second passage, that is, the Master Seed Stock identified as strain Neb-9, was grown in the bacterin medium and lyophilized and stored at 4°C or frozen and stored at -70°C. Strain Neb-9 has been deposited in accordance with the U.S. patent laws and the Budapest Treaty in the American Type Culture Collection, Peoria, Illinois, under accession number 39503.

The Master Seed Stock was confirmed to be a pure culture of gram(-) rods having the following characteristics: autoagglutinated; beta-hemolysis; oxidase (+); gelatin (+); catalase (+); streptomycin resistant; no growth on MacConkey agar; citrate (-); nitrate (-); and phenylalanine (-)

Standard challenge cultures were prepared by growing strain Neb-9 and a heterologous challenge strain, Neb-1, which had been isolated from another calf with IBK, on Trypticase Soy Agar plates containing 0.5% sheep RBC. Plates were incubated for 24 hours at 33°C and then for 4 hours at room temperature. The growth was then removed with a sterile cotton swab and suspended in 1 ml of Trypticase Soy Broth. This was frozen at -70°C as standard challenge seed. One day before calf challenge, the standard challenge culture was thawed. One ml was added to 150 ml of the bacterin medium and grown for 18 hours at 33°C and then for 5 hours at room temperature. The pathogenicity of the challenge was evaluated by infecting eyes of five-six week old calves with different concentrations of M. bovis. The concentration of M. bovis was determined by measuring the O.D. at 590 nm. A needleless tuberculin syringe was used to inoculate 0.5 ml of M. bovis culture under the third lids of both eyes of each calf. Calves were challenged with either M. bovis strain Neb-1 or strain Neb-9. Eyes of calves were examined daily for two weeks for evidence of IBK and then periodically for an additional two weeks.

Extent of disease was measured as follows: A score of 0 indicated that the eye maintained its normal appearance during the observation period. If the eye was lacrimating at anytime during the observation period it received a score of 1. A score of 2 indicated that the eye was swollen (conjunctivitis) in addition to lacrimating; a score of 3 indicated that keratitis in addition to conjunctivitis (IBK) was evident at any time during the observation period. Results are reported in Table 5, below.

TABLE 5

M. bovis Strain	Absorbance 590 nm	Calf No.	Results of Challenge Left Eye	Right Eye
Neb-1	0.15	81	0	0
	0.34	69	3	0
	0.68	64	3	3
	1.5	63	3	3
	1.5	2	3	3
Neb-9	0.34	70	0	3
	0.68	73	3	0

EXAMPLE 2

Bacterin/Protease Vaccine Preparation

A second seed passage of M. bovis strain Neb-9 was grown in the bacterin medium for 24 hours at 33°C to 4.3 absorbance units at 590 nm. Dissolved oxygen was maintained at about 80% or higher by aeration and agitation. The pH was maintained at 7.3 by addition of 5N NaOH. Prior to inactivation with a 1/1200 (0.083%) dilution of beta-propiolactone, the culture was cooled to less than 20°C with constant agitation and the air and exhaust ports were closed.

Prior to inactivation, the viable count of bacteria

in th culture wa[s] [illegible] lon forming units per ml. Inactivation was [illegible] which 7 hours.

Following inact[ivat]ion, merthiolate solution was added to a final concentration of 1/10,000 and Quil A was added to a final concentration of 0.5 mg/ml. The relative protease activity (R.A[illegible]s 1.94

EXAMPLE 3

Efficacy Test: M. bovis Bacterin-Protease Vaccine

Four mixed breed calves 3 to 4 weeks old, were vaccinated with the vaccine described in Example 2. Three mixed breed calves, 3 to 4 weeks old were not vaccinated. The vaccine was administered subcutaneously in the neck region. All vaccinates received 2 doses of the bacterin 21 days apart. Serum was collected for serological testing before vaccination, 21 days following the first vaccination and 7 days following the second vaccination. All calves were challenged with virulent M. bovis.

Table 6 shows that all calves vaccinated with the M. bovis bacterin developed serum agglutinating antibodies by 28 days following vaccination (7 days following the second vaccination). Eyes of calves were examined daily for two weeks for evidence of IBK and then periodically for an additional two weeks. The bacterin, with a RP of 1.10 and an RPA of 0.94 protected 75 percent (3/4) of the vaccinated calves against IBK. All non-vaccinated calves (3/3) developed IBK.

Table 6

Bovine Rotavirus Efficacy Test
Serum Agglutination Titers and Results of Challenge

Vaccination Status	No.	Titer of Agglutination Titers Post Vaccination 21	28	Results of Challenge [I] Left Eye	Right Eye
	3	8	32	0	0
Vaccinated	4	2	64	0	3
	4	4	16	0	0
	4	2	16	0	0
	4	4	4	0	3
Not Vaccinated	4	0	0	3	3
	4	4	2	0	3

[I] Scoring :

0 = Eye was normal, in infection
1 = Eye lacrimating
2 = Conjunctivitis in addition to lacrimation
3 = IBK

EXAMPLE 4

Protease Vaccine Preparation

Supernatant from a second seed passage of M. bovis strain Neb-9 grown in RPMI-640, 2% N-Z Amine A and 0.2% sodium bicarbonate for 2. hours at 33°C to 7.0 absorbance units at 600 nm was concentrated 70X and fractionated on a 15 mm x 90 mm column packed with Bio-Gel p-200 (Bio-Rad) to gain partial separation of a culture component that possessed proteolytic activity and was substantially free of other M. bovis antigens. The eluting buffer was 0.02 M Tris, 0.038 M NaCl and 0.02% NaN_3, pH 8.0. Fractions containing protease activity were pooled and concentrated. The final concentration factor from 500 ml of culture supernatant was 25X. The relative protease activity was 1.0.

Pooled fractions containing the partially purified protease were combined with Quil A at a final concentration of Quil A of 50.ug/ml.

EXAMPLE 5

Mouse Potency Test-Protease Vaccine

The protease vaccine preparation described in Example 4 and a reference bacterin were diluted 5-fold in 0.15 M NaCl containing 50 ug Quil A per ml. Mice (16-20 grams) were vaccinated twice intraperitoneally at 21 day intervals with 0.5 ml of either a 1/10, ~~or~~ 1/50 dilution of the vaccine or the bacterin. Mice were challenged 7 days later with infectious Moraxella bovis strain Neb-1 (31.2 LD_{50}). All survivors in each test dilution were recorded 3 days following challenge. The PD_{50} of the protease vaccine preparation was 1/43.7. The PD_{50} of the reference bacterin was 1/177.2. These results indicate that protease antigens separate from other antigenic components of M. bovis protect mice against M. bovis challenge. Protease antigens separate from other antigenic components will also aid in the protection of

protection of cattle against M. bovis infection. This statement is based on the evidence that there is a direct relationship between the [illegible], RNA of M. bovis bacterins and their effectiveness in protecting cattle against M. bovis infection. The bacterin is more effective, however, than protease antigens alone.

While the preferred embodiments of the invention are described above, it is understood that the invention includes all changes and modifications within the scope of the following claims.

Claims for Contracting States :
BE, CH, DE, FR, GB, IT, LU, NL, SE

1. A vaccine capable of inducing immunity to Moraxella bovis without serious side effects comprising a vaccinal amount of M. bovis protease.

2. The vaccine of claim 1 which is substantially free of other M. bovis antigens.

3. The vaccine of claim 1 which comprises a concentrated fraction of supernatant from a culture of M. bovis grown in a medium which contains a substrate which induces protease production in addition to other nutrients.

4. The vaccine of claim 3 in which the substrate which induces protease production is casein or a casein digest; hyaluronic acid, chondroitin sulfate or other tissue constituents contributing to tissue integrity; yeast extract; beef infusion; or tryptone.

5. The vaccine of claim 3 in which the substrate which induces protease production is casein or a casein digest.

6. The vaccine of claim 3 in which the culture of M. bovis is grown at 30 to 35°C with aeration, at neutral to slightly alkaline pH, until absorbance at 590 nm is at least 2.0 absorbance units.

7. The vaccine of claim 6 in which the culture of M. bovis is grown until absorbance at 590 nm is at least 4.0 absorbance units.

8. The vaccine of claim 6 in which the M. bovis is strain Neb-9.

9. The vaccine of claim 8 in which the medium is RPMI 1640 with 2% N-Z Amine A and 0.2% sodium bicarbonate.

10. A vaccine capable of inducing immunity to M. bovis without serious side effects comprising a vacci-

nal amount of a M. bovis bacterin which contains a component having protective activity.

11. The vaccine of claim 1 in which the amount of protease in the bacterin is sufficient to stimulate an immune response to the protease.

12. The vaccine of claim 1 in which the bacterin is an inactivated culture of M. bovis grown in a medium which contains a substrate which induces protease production in addition to other nutrients.

13. The vaccine of claim 12 in which the substrate which induces protease production is casein or a casein digest; hyaluronic acid; chondroitin sulfate or other tissue constituents contributing to tissue integrity; yeast extract; beef infusion; or tryptone.

14. The vaccine of claim 12 in which the substrate which induces protease production is casein or a casein digest.

15. The vaccine of claim 12 in which the culture of M. bovis is grown at 30 to 35°C with aeration at neutral to slightly alkaline pH, until absorbance at 590 nm is at least 2.0 absorbance units.

16. The vaccine of claim 15 in which the culture of M. bovis is grown until absorbance at 590 nm is at least 4.0 absorbance units.

17. The vaccine of claim 15 in which the M. bovis is strain Neb-9.

18. The vaccine of claim 17 which comprises a culture of M. bovis in RPMI 1640 with 2% N-Z Amine A and 0.2% sodium bicarbonate, which has been inactivated by addition of beta-propiolactone at a final concentration of 1:1200 and adjuvanted with Quil A to a final concentration of Quil A of 0.1 mg/ml.

Claims for the Contracting State AT

1. A process for preparing a vaccine capable of inducing immunity to Mycoplasma bovis without serious side effects comprising growing M. bovis in a medium which contains a substrate which induces protease production in addition to other nutrients, isolating and concentrating the antigen thus obtained.

2. A process according to claim 1 wherein the obtained vaccine is substantially free of other M. bovis antigens.

3. The process of claim 1 or 2 in which the substrate which induces protease production is casein or a casein digest; hyaluronic acid, chondroiten sulfate or other tissue constituents contributing to tissue integrity; yeast extract; beef infusion; or tryptone.

4. The process of claim 1 or 2 in which the substrate which induces protease production is casein or a casein digest.

5. The process of any of claims 1-4 in which the culture of M. bovis is grown at 30 to 35°C with aeration, at neutral to slightly alkaline pH, until absorbance at 590 nm is at least 2.0 absorbance units.

6. The process of any of claims 1-5 - in which the culture of M. bovis is grown until absorbance at 590 nm is at least 4.0 absorbance units.

7. The process of any of claims 1-6 in which the M. bovis is strain Neb-9

8. The process of any of claims 1-7 in which the medium is RPMI 1640 with 2% N-Z Amine A and 0.2% sodium bicarbonate.

9. A process for preparing a vaccine capable of inducing immunity to M. bovis without serious side effects comprising growing M. bovis in a medium which contains a substrate which induces protease production

in addition to other supplements and inactivating the culture medium.

10. A process according to claim 8 in which the substrate which induces protease production is casein or a casein digest; hyaluronic acid, chondroitin sulfate or other tissue supplements contributing to tissue integrity; yeast extract; beef infusion; or tryptone.

11. The process of claim 9 in which the substrate which induces protease production is casein or a casein digest.

12. The process of any of claims 8-10 in which the culture of M. bovis is grown at 34 to 35°C with aeration at neutral to slightly alkaline pH, until absorbance at 590 nm is at least 2.0 absorbance units.

13. The process of any of claims 8-11 in which the culture of M. bovis is grown until absorbance at 590 nm is at least 4.0 absorbance units.

14. The process of any of claims 8-12 in which the M. bovis is strain Neb-9.

15. The process of any of claims 8-13 in which the medium is RPMI 1640 with 4% N-Z Amine and 0.2% sodium bicarbonate and the inactivation is performed by addition of beta-propiolactone at a final concentration of 1:1200, the inactivated medium being adjuvanted with Quil A to a final concentration of Quil A of 0.5 mg/ml.